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Killing of Feline T-Lymphocytes by Gamma-Rays and Energetic Carbon Ions

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ABSTRACT. High linear energy transfer (LET) heavy charged particles have previously been applied clinically to human cancer radiotherapy because of their excellent physical properties of selective dose distribution and higher relative biological effectiveness (RBE) for human; however, such an approach has yet to be applied to cat patients. The present study investigates the biological effectiveness of low-LET γ-rays (0.2 keV/μm) compared to high-LET carbon ions (114 keV/μm) in feline T- lymphocyte FeT-J cells. Clonogenic survival analysis revealed that the RBE value of carbon ions was 2.98 relative to a 10% survival dose (D_{10}) by γ-rays, and that the inactivation cross-section in cells exposed to γ-rays and carbon ions was 0.023 and 38.9 μm^2, respectively. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) analysis revealed that TUNEL-positive frequency in carbon-irradiation cells is higher than for γ-irradiated cells against exposure to the same physical doses, but that very little difference in TUNEL-positive frequency is observed between cells exposed to the respective D_{10} dose of γ-rays. Our data thus indicate that carbon ions are more effective for cell killing than γ-rays at the same physical doses, but kill cells to an extent that is comparable to γ-rays at the same biological doses. Carbon ion radiotherapy is therefore a promising modality for cat patients.

KEY WORDS: accelerated carbon ions, apoptosis, feline T lymphocytes, gamma-ray, relative biological effect.

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Ionizing radiations, such as X-rays and γ-rays, have been clinically applied to the treatment of cat patients with immunosuppression by whole-body irradiation prior to bone marrow transplantation [5] and to the treatment of primary intratracheal lymphosarcoma and oral squamous cell carcinoma, and have achieved complete remission [4, 10].

Many lines of evidence have shown that biological effectiveness in human and rodent cells differs with the linear energy transfer (LET) of ionizing radiations. High-LET radiations, including energetic heavy-ion particles, are more effective for cell killing [6, 18, 27] and apoptosis [20, 24] than low-LET photons such as X-rays and γ-rays. Relative biological effectiveness (RBE) values reach a maximum at a LET of 100–200 keV/μm [1, 3, 8, 22]. High-LET heavy ions have a more selective dose distribution as well as a higher RBE, and have been applied to radiotherapy in human patients for the treatment of malignant tumors in the lung [11], brain [12], liver [15], and prostate tissues [26]; this treatment has been shown to be more effective than therapy using conventional low-LET photons [25]. Although it is highly likely that heavy-ion radiotherapy is a promising modality, the application of heavy ions in veterinary clinics is unreported. No studies have reported the killing effect of heavy ions in companion animals such as cats, while many data are available for human and rodent cells.

This study aims to provide basic insights into the application of heavy ions to radiotherapy in cat patients by comparison of the susceptibility of feline cells to high- and low-LET radiation.

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MATERIALS AND METHODS

Cell Cultures and Irradiation: The feline T-lymphocyte FeT-J cells (CD4+, CD8±) used in this study were obtained from the American Type Culture Collection (ATCC CRL11967) [9, 29]. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2-in-air and routinely subcultured every 4 days in RPMI 1640 growth medium (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Equitech-Bio, Kerrville, TX, U.S.A.) and 0.1 μg/ml kanamycin (Invitrogen). For irradiation experiments, 65 μl cell suspension was added to Slide Seal for *in situ* PCR (1.5 × 1.6 cm, Takara, Tokyo, Japan) and covered with 8 μm thick Kapton polyimide film (Toray-DuPont, Tokyo, Japan) to prevent air drying in non-coated 60 mm non-treated plastic dishes (Asahi Techno Glass, Chiba, Japan). The cells were exposed at room temperature to either 0–8 Gy of γ-rays (0.2 keV/μm) from a ^{60}Co source at a dose rate of 2 Gy/min, or to 0–2 Gy of energetic carbon ions (220 MeV ^{12}C, 114 keV/μm) delivered from the azimuthally-varying-field (AVF) cyclotron installed at TIARA (Takasaki Ion Accelerators for Advanced Radiation Application) facilities at the Japan Atomic Energy Agency (JAEA) [8]. We calculated the LET value based on the kinetic energy loss assuming water equivalence, and the absorbed dose (Gy) was calculated according to the formula

Dose (Gy) = fluence (number of ion particles/cm²) × LET (keV/μm) × 1.6 × 10⁻⁹ [8, 16]. Sham-irradiated control cells were manipulated in parallel with the test cells. After irradiation, cells were held at 37°C and 5% CO_2 for the desired period of time until analysis.

Clonogenic Survival Assay: Cell survival was tested using a clonogenic survival assay. Irradiated cells were replated in 2 m*l* of medium that contained 0.2% SeaKem® GTG Agarose (Takara Bio, Shiga, Japan) in non-tissue culture plastic dishes. Three milliliters of medium containing 0.4% agarose was used as a basal layer. On day 14, colonies containing more than 50 cells were scored as survivors [21]. We calculated surviving fractions based on the plating efficiency of control cells, and survival curves were fitted against the means of three (γ-rays) or four (carbon ions) independent experiments with triplicate measurements to the exponential equation $y = exp(-ax)$, where y, x, and a are the surviving fraction, dose and slope, respectively. D_{10}, the dose needed to reduce the surviving fraction in colonies to 0.1, was calculated as $[ln(1/0.1)]/a$. Mean lethal dose (D_0), the dose required to decrease the surviving fraction by e, was calculated as $[ln(0.37)^{-1}/a]$. RBE values were calculated as a fraction of the D_{10} dose for carbon ions divided by that for γ-rays. Inactivation cross-sections (μm²) were calculated as *LET (keV/μm) × 0.16021/D_0 (Gy)* [8, 27].

Detection of Apoptosis with TUNEL Assay: Apoptosis induction was analyzed up to 4 days post-irradiation using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method [7]. Irradiated cells were washed once with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS^-), followed by fixation with 1% paraformaldehyde in PBS^- for 10 min at room temperature. Cells were then dropped onto an MAS-coated glass slide (Matsunami, Osaka, Japan) and air-dried. TUNEL assay was performed with ApopTag® plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, U.S.A.) according to the manufacturer's instructions. Briefly, slides were washed twice with PBS-, and endogenous peroxidase was inactivated by a 5 min reaction with 3% hydrogen peroxide. Slides were pretreated for 10 sec with TdT equilibration buffer after washing twice with PBS^-, before being treated with TdT enzyme for 60 min at 37°C in a humidified chamber. After washing three times with PBS^-, slides were reacted with peroxidase-conjugated anti-digoxigenin antibodies for 30 min at 37°C in a humidified chamber. After washing four times with PBS^-, the positive staining of peroxidase binding was visualized using diaminobenzidine as a peroxidase substrate. Cell nuclei were counterstained with 0.5% methyl green for 10 min at room temperature. We observed the cells under a light microscope (BX60, Olympus, Tokyo, Japan); more than 1,000 cells were analyzed in each sample to evaluate apoptosis frequency.

Statistical Analysis: Comparisons between groups were made by Student's t-test and a p value of 0.05 or less between groups was considered significant.

RESULTS

To compare the killing effects of low-LET γ-rays and high-LET carbon ions (properties listed in Table 1), irradiated FeT-J cells were subjected to clonogenic analyses. Irradiation with carbon ions used in this study is expected to be most effective for cell killing, as it has been shown for human and rodent cells that RBE peaks at 100–200 keV/μm [1, 3, 8, 22]. Carbon-ion irradiation significantly decreased the clonogenicity of FeT-J cells compared to γ-rays, as shown in Fig. 1. This indicates a higher sensitivity to carbon ions.

Induction of apoptosis by ionizing radiations in human and rodent lymphocytes occurs more frequently than that in other tissues [2]. Therefore, to compare apoptosis frequency, irradiated cells were subjected to TUNEL assay. As is apparent in Fig. 2 (left and middle panels), there was no difference in TUNEL-positive frequency ($p>0.05$) with variable exposure to D_{10} doses of γ-rays and carbon ions. In contrast, we observed a lower apoptosis frequency in cells exposed to 1.2 Gy of γ-rays approximately corresponding to the D_{10} dose of carbon ions than was observed in cells exposed to 3 Gy of γ-rays corresponding to the D_{10} dose of γ-rays (middle and right panels in Fig. 2). Compared with the TUNEL-positive frequency in sham-irradiated control cells, we observed statistically significant differences ($p<0.05$) on days 2 and 4 in cells exposed to D_{10} doses of

Table 1. Parameters of radiosensitivity of FeT-J cells

Radiation	LET (keV/μm)	D_{10}[a] (Gy)	D_0[b] (Gy)	RBE[c]	Inactivation cross section[d] (μm²)
^{60}Co γ-rays	0.2	3.25	1.40	1.00	0.023
220 MeV ^{12}C	114	1.09	0.47	2.98	38.9

Notes. Survival curve of FeT-J exposed to γ-rays and carbon ion were calculated according to the following equation, $y=exp(-ax)$ where y, x and a are surviving fraction, dose and slope, respectively.

a) The dose giving 10% survival (D_{10}) was calculated as $[ln(0.1)^{-1}]/a$.

b) Mean lethal dose (D_0), the dose required to decrease surviving fraction by e (base of natural logarithms) i.e. 37 % survival, was calculated as $[ln(0.37)^{-1}/a]$.

c) Relative biological effectiveness (RBE) was calculated as fraction of D_{10} dose of carbon ion divided by that of γ-rays.

d) Inactivation cross section was calculated as *LET (keV/μm) × 0.1602/D_0 (Gy)*.

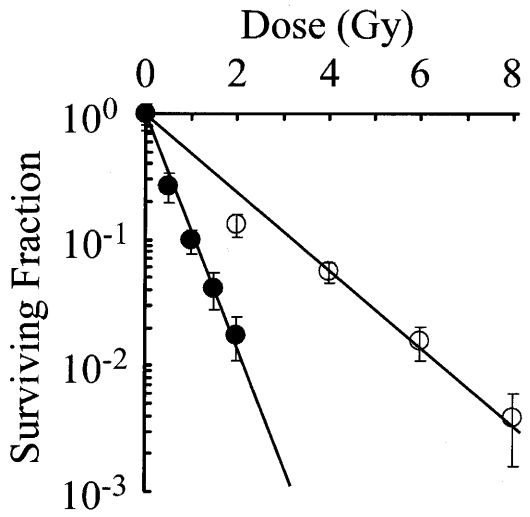

Fig. 1. Susceptibility of FeT-J cells to γ-rays and carbon ions. Cells irradiated with the indicated doses of γ-rays were subjected to clonogenic survival assay. Open and closed circles represent the clonogenic survival of FeT-J exposed to γ-rays and carbon ions, respectively. These data represent the means and standard errors of three (γ-rays) or four (carbon ions) independent experiments with triplicate measurements.

carbon ion, and on days 2, 3, and 4 in cells exposed to the D_{10} dose as well as to 1.2 Gy of γ-rays. On day 4, TUNEL-positive frequency observed in cells exposed to the D_{10} doses of γ-rays and carbon ions was statistically different to that in cells exposed to 1.2 Gy of γ-rays. These data suggest that apoptosis induction increases in a time- and dose-dependent manner but are comparable irrespective of LET in cells exposed to biologically equivalent doses such as D_{10} doses.

DISCUSSION

In this study, we have demonstrated for the first time the susceptibility of feline cells to high-LET heavy ions in terms of clonogenic survival and apoptosis induction. Irradiation with energetic carbon ions (114 keV/μm), of which LET has been known to maximize biological effects in human and rodent cells [1, 3, 8, 22], exacerbated the killing of feline T-lymphocyte FeT-J cells with RBE of 2.98 relative to the D_{10} dose of g-rays (0.2 keV/μm). Inactivation cross-section in cells exposed to carbon ions was 1691-fold higher than that in γ-irradiated cells (Fig. 1 and Table 1). A dose-dependent increase in apoptosis induction was found in γ-irradiated cells, and apoptosis induction by carbon ions was higher than that by γ-rays at physically equivalent doses, whereas apoptosis induction by γ-rays and carbon ions was comparable at biologically equivalent D_{10} doses (Fig. 2).

Cats are less likely than dogs to develop acute radiation responses at comparable doses [19]. Feline lymphocytes show a lower frequency of X-ray induced chromosome aberrations, such as dicentrics, than human lymphocytes [23]. Therefore, it is likely that radiation responses of feline lymphocytes are distinct from those of the lymphocytes of other species [19, 23]. Here, we found that the D_0 dose of FeT-J cells for γ-rays was 1.40 Gy (Table 1). It has been reported that D_0 doses for low-LET photons are 1.2 Gy in canine multi-potent hemopoietic progenitor cells [17], 1.95 Gy in murine T-lymphocytes [14], and 2 Gy in murine B-lymphocytes [14]. Inactivation cross-section in FeT-J cells exposed to carbon ions is greater than that in CHO-K1 ovarian cells of the Chinese hamster [18] and normal human fibroblast AG01522 cells [8]. FeT-J cells are likely to be more radiosensitive than CHO-K1 cells and human malignant melanoma HMV-II, and less than murine lymphoma L5178Y [28].

While low-LET photons pass through the entire body, high-LET radiation such as heavy ions forms a Bragg peak

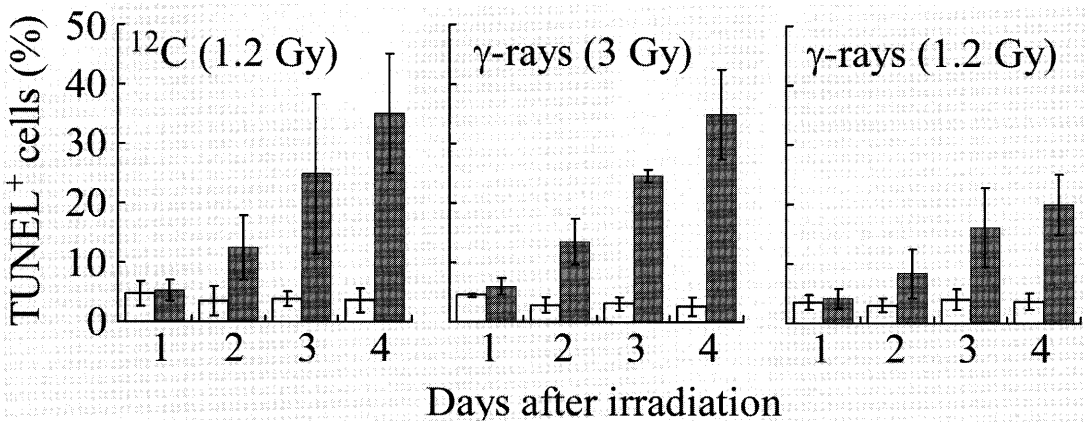

Fig. 2. Induction of apoptosis by γ-ray and carbon-ion irradiation. FeT-J cells exposed to carbon ions or γ-rays were incubated at 37°C for the stated time, followed by TUNEL assay. Open and shaded columns represent the TUNEL-positive fraction in sham controls and irradiated samples, respectively. These data are presented as means and standard errors of three independent experiments where >1000 cells were analyzed in each experiment.

and deposits a maximum energy at a certain depth. Typically, carbon ions (220 MeV ^{12}C) used in the present study reach the Bragg peak at 1.094 mm, where the value was calculated according to the kinetic energy loss assuming water equivalence. Moreover, by using a ridge filter, the Bragg peak can be spread out such that deep-seated tumors can be selectively irradiated with minimal exposure to surrounding normal tissues. In addition to this excellent selectivity of special dose distribution, heavy ions with a LET of 100–200 keV/μm are most effective for cell killing [1, 3, 8, 22]. Furthermore, hypoxic cells within cancerous tissues, which are resistant to low-LET photons, can be effectively killed by high-LET heavy ions. Therefore, heavy-ion radiotherapy with a selective target region is highly likely to minimize side effects and maximize the efficiency of therapy. This suggests that heavy-ion radiotherapy is a most promising modality in veterinary clinics, including the treatment of cat patients.

In conclusion, this study is the first to demonstrate that carbon ions are more effective for killing feline cells than γ-rays at the same physical doses, but kill cells to an extent that is comparable to γ-rays at the same biological doses. Our present findings provide basic insights into the application of heavy ions in veterinary clinics. We recently studied the death modes against γ-rays in two lines of feline T-lymphocytes and found that apoptosis dominated in one line but non-apoptotic mitotic catastrophe dominated in the other [13]. This indicates that the modes of radiation-induced cell death are distinct, even between feline T-lymphocytes. The application of heavy ions within veterinary clinics requires further studies to carefully examine the radiosensitivity of many types of malignant and normal cells to heavy ions over wide-ranging LET values.

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REFERENCES

1. Barendsen, G. W., Walter, H. M., Fowler, J. F. and Bewley, D. K. 1963. Effects of different ionizing radiations on human cells in tissue culture. III. Experiments with cyclotron-accelerated alpha-particles and deuterons. *Radiat. Res.* **18**: 106–119.
2. Belka, C., Marini, P., Budach, W., Schulze-Osthoff, K., Lang, F., Gulbins, E. and Bamberg, M. 1998. Radiation-induced apoptosis in human lymphocytes and lymphoma cells critically relies on the up-regulation of CD95/Fas/APO-1 ligand. *Radiat. Res.* **149**: 588–595.
3. Broerse, J. J., Barendsen, G. W. and van Kersen, G. R. 1968. Survival of cultured human cells after irradiation with fast neutrons of different energies in hypoxic and oxygenated conditions. *Int. J. Radiat. Biol.* **13**: 559–572.
4. Brown, M. R., Rogers, K. S., Mansell, K. J. and Barton, C. 2003. Primary intratracheal lymphosarcoma in four cats. *J. Am. Anim. Hosp. Assoc.* **39**: 468–472.
5. Cain, J. L., Cain, G. R., Turrel, J. M. and Theilen, G. H. 1990. Clinical and lymphohematologic responses after bone marrow transplantation in sibling and unrelated donor-recipient pairs of cats. *Am. J. Vet. Res.* **51**: 839–844.
6. Fournier, C., Scholz, M., Weyrather, W. K., Rodemann, H. P. and Kraft, G. 2001. Changes of fibrosis-related parameters after high- and low-LET irradiation of fibroblasts. *Int. J. Radiat. Biol.* **77**: 713–722.
7. Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. 2001. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* **119**: 493–501.
8. Hamada, N., Funayama, T., Wada, S., Sakashita, T., Kakizaki, T., Ni, M. and Kobayashi, Y. 2006. LET-dependent survival of irradiated normal human fibroblasts and their descendents. *Radiat. Res.* **166**: 24–30.
9. Hohdatsu, T., Hirabayashi, H., Motokawa, K. and Koyama, H. 1996. Comparative study of the cell tropism of feline immunodeficiency virus isolates of subtypes A, B and D classified on the basis of the env gene V3-V5 sequence. *J. Gen. Virol.* **77**: 93–100.
10. Jones, P. D., de Lorimier, L. P., Kitchell, B. E. and Losonsky, J. M. 2003. Gemcitabine as a radiosensitizer for nonresectable feline oral squamous cell carcinoma. *J. Am. Anim. Hosp. Assoc.* **39**: 463–467.
11. Kadono, K., Homma, T., Kamahara, K., Nakayama, M., Satoh, H., Sekizawa, K. and Miyamoto, T. 2002. Effect of heavy-ion radiotherapy on pulmonary function in stage I non-small cell lung cancer patients. *Chest* **122**: 1925–1932.
12. Kageji, T. and Nagahiro, S. 2005. Present status of radiotherapy for malignant glioma. *Jpn. J. Neursurg.* **14**: 121–131.
13. Kakizaki, T., Hamada, N., Wada, S., Funayama, T., Sakashita, T., Hohdatsu, T., Sano, T., Natsuhori, M., Kobayashi, Y. and Ito, N. 2006. Distinct modes of cell death by ionizing radiation observed in two lines of feline T-lymphocytes. *J. Radiat. Res.* (in press).
14. Kataoka, Y. and Sado, T. 1975. The radiosensitivity of T and B lymphocytes in mice. *Immunology* **29**: 121–130.
15. Kato, H., Tsujii, H., Miyamoto, T., Mizoe, J. E., Kamada, T., Tsuji, H., Yamada, S., Kandatsu, S., Yoshikawa, K., Obata, T., Ezawa, H., Morita, S., Tomizawa, M., Morimoto, N., Fujita, J. and Ohto, M. 2004. Results of the first prospective study of carbon ion radiotherapy for hepatocellular carcinoma with liver cirrhosis. *Int. J. Radiat. Oncol. Biol. Phys.* **59**: 1468–1476.
16. Kraft, G. 2000. Tumor therapy with heavy charged particles. *Prog. Part. Nucl. Phys.* **45**: 473–544.
17. Kreja, L., Weinsheimer, W. and Nothdurft, W. 1991. *In vitro* studies on the radiosensitivity of multipotent hemopoietic progenitors in canine bone marrow. *Exp. Hematol.* **19**: 755–758.
18. Mehnati, P., Morimoto, S., Yatagai, F., Furusawa, Y., Kobayashi, Y., Wada, S., Kanai, T., Hanaoka, F. and Sasaki, H. 2005. Exploration of "over kill effect" of high-LET Ar- and Fe-ions by evaluating the fraction of non-hit cell and interphase death. *J. Radiat. Res.* **46**: 343–350.
19. Moore, A. S. 2002. Radiation therapy for the treatment of tumours in small companion animals. *Vet. J.* **164**: 176–187.
20. Otto, S. M., Vazquez, E. and Guida, P. 2005. Cytotoxic effects of low- and high-LET radiation on human neuronal progenitor cells: induction of apoptosis and TP53 gene expression. *Radiat. Res.* **164**: 545–551.
21. Puck, T. T. and Marcus, P. I. 1956. Action of x-rays on mam-

malian cells. *J. Exp. Med.* **103**: 653–666.
22. Rodriguez, A., Alpen, E. L. and Powers-Risius, P. 1992. The RBE-LET relationship for rodent intestinal crypt cell survival, testes weight loss, and multicellular spheroid cell survival after heavy-ion irradiation. *Radiat. Res.* **132**: 184–192.
23. Stephan, G., Adler, I. D., Schwartz-Porsche, D., Hollihn, K. U. and Obe, G. 1979. Characterization of feline whole-blood cultures and determination of the frequency of radiation-induced dicentrics in human and feline lymphocytes. *Int. J. Radiat. Biol.* **35**: 351–359.
24. Takahashi, A., Matsumoto, H., Yuki, K., Yasumoto, J., Kajiwara, A., Aoki, M., Furusawa, Y., Ohnishi, K. and Ohnishi, T. 2004. High-LET radiation enhanced apoptosis but not necrosis regardless of p53 status. *Int. J. Radiat. Oncol. Biol. Phys.* **60**: 591–597.
25. Tsujii, H. and Yanagi, T. 2005. Heavy ion radiotherapy. *Biotherapy* **19**: 103–113.
26. Tsujii, H., Mizoe, J. E., Kamada, T., Baba, M., Kato, S., Kato, H., Tsuji, H., Yamada, S., Yasuda, S., Ohno, T., Yanagi, T., Hasegawa, A., Sugawara, T., Ezawa, H., Kandatsu, S., Yoshikawa, K., Kishimoto, R. and Miyamoto, T. 2004. Overview of clinical experiences on carbon ion radiotherapy at NIRS. *Radiother. Oncol.* **73**: 41–49.
27. Tsuruoka, C., Suzuki, M., Kanai, T. and Fujitaka, K. 2005. LET and ion species dependence for cell killing in normal human skin fibroblasts. *Radiat. Res.* **163**: 494–500.
28. Wada, S., Kurahayashi, H., Kobayashi, Y., Funayama, T., Yamamoto, K., Natsuhori, M. and Ito, N. 2003. The relationship between cellular radiosensitivity and radiation-induced DNA damage measured by the comet assay. *J. Vet. Med. Sci.* **65**: 471–477.
29. Yamamoto, J. K. 1998. Feline derived T-cell lines for producing FIV. *U.S. Patent* 5,846,825.